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Original Article

Tenascin-C is a diagnostic marker for cervical cancer and a potential marker for cancer-associated fibroblasts in cervical carcinoma

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Abstract: Background: Tenascin-C (TNC) is an extracellular matrix protein that is involved in various tissue interactions during fetal development and oncogenesis. In the present study, we clarified the role of TNC in cervical carcinoma by exploring the associations between TNC and cancer-associated fibroblast (CAF) markers such as fibroblast-stimulating protein-1 (FSP1), smooth muscle actin (SMA), and vimentin. Methods: We investigated the expression of TNC, human papillomavirus 16 and 18 early protein 6 (HPV 16/18 E6), FSP1, SMA, vimentin, CD163, and CD34 in 166 cervical carcinoma tissue samples using immunohistochemistry. Statistical analysis was performed to compare the rates of individual protein expression with the clinicopathological parameters and to study the association between two individual protein markers (e.g., TNC/HPV-16/18 E6, TNC/FSP1, TNC/SMA, TNC/vimentin, and TNC/CD163). Results: TNC, FSP1, SMA, vimentin, and CD163 expression was higher in cervical cancer tissues than in non-neoplastic cervical epithelium. TNC expression was found to be associated with HPV-16/18 E6, clinical stage, lymphovascular invasion, and microvessel density, whereas no association was found with other clinicopathological features of the stroma of cervical carcinoma. However, TNC, SMA, vimentin, FSP1, and CD163 were negatively correlated with overall survival at the mRNA level. In addition, TNC was also correlated with SMA, FSP1, and CD163, but not with vimentin. Conclusions: Overexpression of TNC in cervical carcinoma might serve as a novel CAF marker of the disease.

Keywords: Tenascin-C, diagnostic, CAF, cervical carcinoma, biomarker

Introduction

Cervical carcinoma is the third leading cause of cancer-related death in women worldwide. Cervical cancer cells require a laminin-rich fibrillar matrix for their invasion, creating an imbalance between various components of the basement membrane [1]. The tumor microenvironment is characterized by a stroma and tumor cells that express a tumor-specific extracellular matrix (ECM), growth factors, cytokines, and matrix remodeling enzymes. The major cellular component of the tumor stroma is cancer-associated fibroblasts (CAFs), which act as an essential microenvironment for cancer cells and express TNC and other proteins, hence promoting tumor progression, invasion, and metastasis [2]. Tenascin-C (TNC) is an ECM glycoprotein composed of hexamers of 200-400 kDa subunits. It is transiently expressed during organogenesis and in pathologic conditions including inflammation and cancer [3]. In particular, TNC is highly expressed in the tumor microenvironment of solid malignancies including lung cancer, breast cancer, colon cancer, glioblastoma [4], hepatocellular carcinoma (HCC) [5], and esophageal squamous cell carcinoma (ESCC) [6]. TNC has been suggested to act as a biomarker for HCC recurrence and metastasis and as a potential target for HCC therapy [5].

The aim of the present study was to investigate the role of TNC in cervical cancer diagnosis as well as the identification of a novel CAF marker for cervical carcinoma as a potential anticancer target, exploiting the corresponding tumor-specific ECM by using logistic regression and immunohistochemical analysis.

Materials and methods

Ethics statement

This research complied with the Helsinki Declaration and was approved by the Human Ethics Committee and the Research Ethics Committee of Yanbian University. All patients provided written informed consent according to institutional guidelines. Patients were informed that the resected specimens were stored by the hospital and might potentially be used for scientific research, and that their privacy would be maintained. Follow-up survival data were collected retrospectively through medical record analyses.

Tissue specimens

A total of 166 formalin-fixed and paraffin-embedded tumor tissue samples including 75 cervical carcinoma, 57 cervical intraepithelial neoplasia, and 34 adjacent non-tumor cervical mucosa samples were obtained from the Department of Pathology at Yanbian University in accordance with protocols approved by the Institutional Review Board. No patient received preoperative chemotherapy or radiotherapy. Clinical and pathological reports were reviewed for age, sex, tumor size, histological grade, invasion depth, nodal status, and distant metastasis. The pathological tumor, node, metastasis (pTNM) classification was applied according to guidelines from the 2010 American Joint Committee on Cancer staging manual (AJCC 7th edition).

Immunohistochemical staining procedure

Sections on microslides were deparaffinized with xylene, hydrated using a diluted alcohol series, and immersed in 3% H_2O_2 in methanol to quench endogenous peroxidase activity. Sections were treated with TE buffer (10 mM Tris and 1 mM EDTA, pH 9.3) at 98°C for 30 min. To reduce non-specific staining, each section was blocked with 4% bovine serum albumin in PBS with 0.1% Tween 20 for 30 min. The sections were then incubated with the following monoclonal antibodies: anti-TNC (1:100, Abcam, Cambridge, UK), anti-SMA (1:100, ZSGB-BIO, China), anti-vimentin (1:100, ZSGB-BIO, Beijing, China), anti-FSP1 (1:100, ZSGB-BIO), anti-CD163 (1:100, ZSGB-BIO), and anti-HPV 16/18 E6 (1:100, Abcam) in PBST containing 3 mg/ml goat globulin (Sigma, St. Louis, MO, USA) for 60 min at room temperature, followed by three successive washes with buffer. The chromogen (ImmPACT AEC Peroxidase Substrate, VECTOR Laboratories, Burlingame, CA, USA) was applied for 20 min. Sections were counterstained with Meyer's hematoxylin. After photographing the slide (Model: ECLIPSE Ni-U; Manufacturer: Nikon; Location: Japan), the sections were then subjected to stripping buffer (20% SDS, 0.5 M Tris, and mercaptoethanol) to remove the original antibody for 1 h in a water bath at 56°C and then for 10 min in dehydrated alcohol to removing the red reaction, so that the sections could be used again. Negative controls for immunostaining were generated by omitting the primary antibody.

The double immunostaining procedure was performed using a two-step method with anti-TNC or anti-SMA antibodies (developed with 3, 3'-diaminobenzidine) (brown reaction product) and anti-CD34 (1:100, ZSGB-BIO) or anti-HPV 16/18 E6 antibodies (developed with ImmPACT AEC Peroxidase Substrate) (red reaction product) to observe the relationship between the expression of TNC/SMA and MVD, TNC, and HPV 16/18 E6 in cervical carcinoma, respectively. The protocols for the TNC and SMA detection were essentially as described, except that the chromogen with the 3, 3'-diaminobenzidine (Dako, Glostrup, Denmark) was applied for 10 min. Then, subsequent staining of the same section was performed after incubating the samples with an antibody to CD34 or HPV 16/18 E6 followed with detection by ImmPACT AEC Peroxidase Substrate for 20 min.

Two pathologists (YHX & ZTY) who did not possess knowledge of the clinical data examined and scored all tissue specimens. CD34 positive individual microvessel counts were performed on 200× fields and three area microvessel numbers were averaged as the microvessicle density (MVD). In case of discrepancies, a final score was established by reassessment by both pathologists on a double-headed microscope. As described in detail previously, the staining results were semi-quantitatively scored as negative and positive [6].

Immunofluoresce imaging intensity assay

Sections on microslides were deparaffinized with xylene, hydrated using a diluted alcohol

Table 1. Association of TNC, CAF markers, and CD163 with carcinogenesis of cervical tissue

Diagnosis	*N*	TNC (+) *n* (%)	χ^2	*P*	FSP1 (+) *n* (%)	χ^2	*P*	SMA (+) *n* (%)	χ^2	*P*	vimentin (+) *n* (%)	χ^2	*P*	CD163 (+) *n* (%)	χ^2	*P*
NNL	34	7 (20.6)	19.497	<0.001	3 (8.8)	32.989	<0.001	4 (11.8)	33.019	<0.001	8 (24.2)	14.294	0.003	12 (35.3)	19.553	<0.001
CIN 1-2	9	5 (55.6)			5 (55.6)			2 (22.2)			4 (44.4)			4 (44.4)		
CIN 3	48	28 (58.3)			28 (59.6)			20 (41.7)			21 (43.8)			32 (66.7)		
SCC	75	49 (65.3)			50 (66.7)			51 (68.0)			47 (62.7)			43 (57.3)		

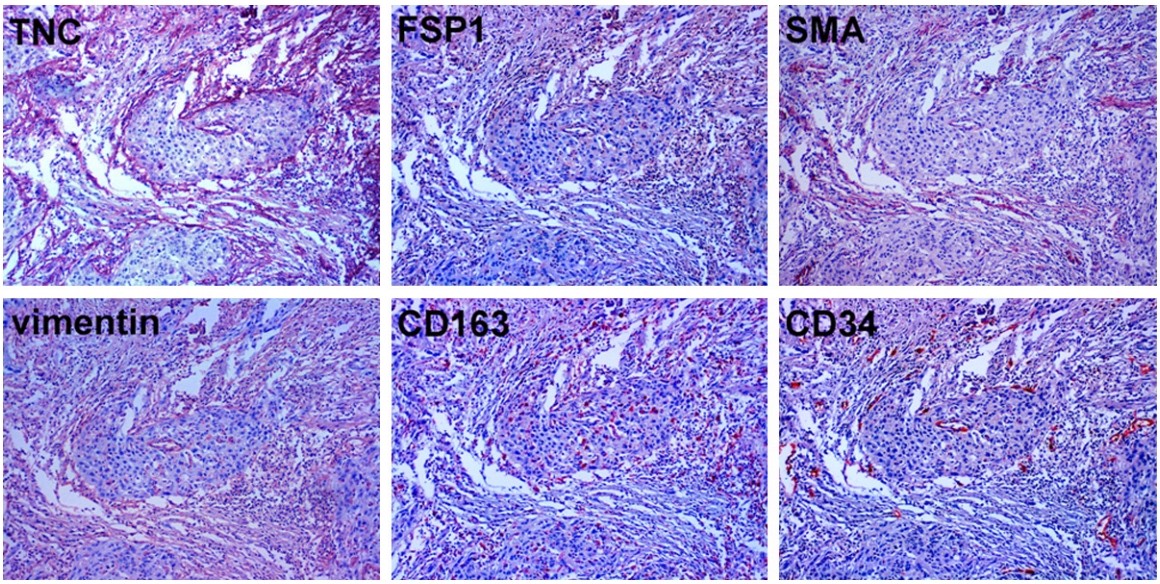

Figure 1. Immunohistochemical staining of cervical carcinoma with TNC, FSP1, SMA, vimentin, CD163, and CD34 in the same field.

series, and immersed in 3% H_2O_2 in methanol to quench endogenous peroxidase activity. Sections were treated with TE buffer (10 mM Tris and 1 mM EDTA, pH 9.3) at 98°C for 30 min. To reduce non-specific staining, each section was blocked with 2% FBS and 1% BSA in PBS for 30 min. The sections were then incubated with primary antibodies against TNC (1:100, Abcam), FSP1 (1:100, ZSGB-BIO) and SMA (1:100, ZSGB-BIO) overnight at 4°C. The next day, cells were incubated with Alexa Fluor 568 goat anti-mouse IgG (Invitrogen, A12380) and Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, A11008) secondary antibodies (1:500 dilution) for 1 hr. Nuclei were stained with DAPI and sections were mounted with vectorshield mounting medium with DAPI for fluorescence detection (vector lab, H-1200). Fluorescence detection was performed with the Axiovert 200 II (Carl-Zeiss). The intensity of immunofluorescence of cells was measured using MetaMorph software.

Statistical analysis

Correlations were examined using Pearson's chi-square test as appropriate. All tests were two sided, and a *p*-value of less than 0.05 was considered statistically significant. The statistical analysis was performed using SPSS statistical software (SPSS Inc., Chicago, IL, USA).

Results

Association of TNC and CAF marker expression with clinicopathological characteristics

FSP1, SMA, and vimentin have been identified as conventional CAF markers [7, 8]. TNC (χ^2=19.497, *P*<0.001), FSP1 (χ^2=32.989, *P*<0.001), SMA (χ^2=33.019, *P*<0.001), vimentin (χ^2=14.294, *P*=0.003), and CD163 (χ^2=19.553, *P*<0.001) expression levels were significantly higher in cervical cancer tissues than in non-neoplastic cervical epithelium (**Table 1**). Positive signals of TNC and CAF markers were mainly localized in stromal fibroblasts, whereas CD163 was expressed in the cell membrane of tumor-associated macrophages (TAMs) (**Figure 1**).

Then we studied the clinical relevance of TNC and TNC associated CAF markers in cervical carcinoma by comparing the level of their protein expression with clinic-pathologic parameters (**Figure 2**). TNC expression was associated with clinical stage (χ^2=4.945, *P*=0.026) and lymphovascular invasion (LVI) (χ^2=5.920, *P*=0.015) in the stroma of cervical carcinoma. HPV-16/18 E6 was upregulated in cervical cancer tissue whereas TNC expressed in the surrounding stroma; notably, HPV-16/18 E6 was positively correlated with TNC. No significant association of TNC expression was observed

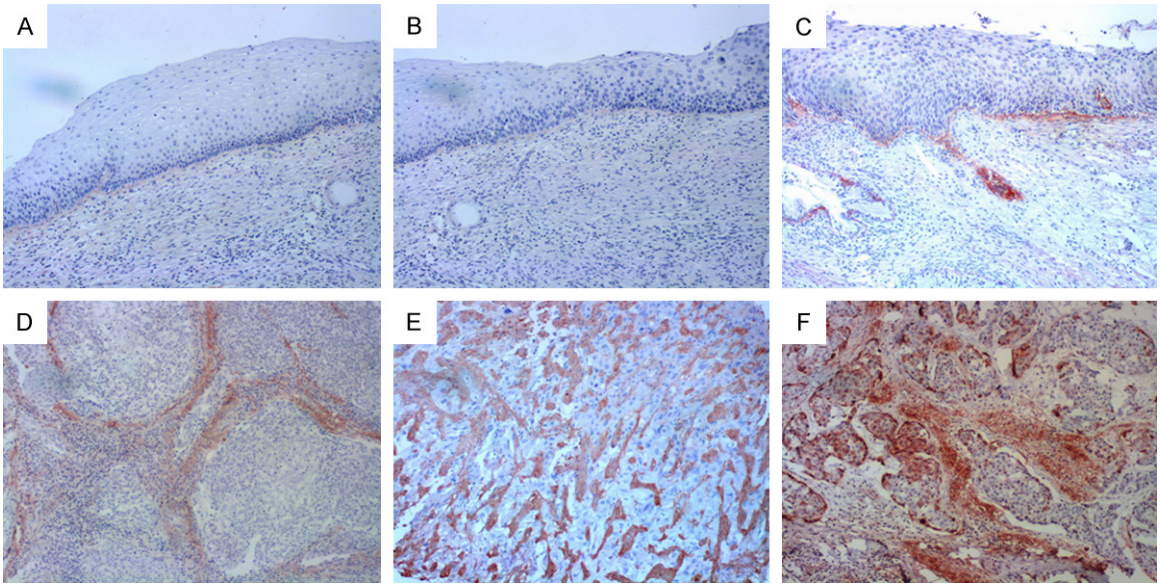

Figure 2. Immunohistochemical staining of human TNC in normal cervix, cervical intraepithelial neoplasia, and cervical carcinoma differentiated to different degrees. Representative images show immunohistochemical staining for TNC expression in (A) undifferentiated normal, (B, C) well-differentiated pre-cancerous stages of cervical intraepithelial neoplasia (CIN) II and CIN III, (D) moderately differentiated CSCCs, (E) poorly differentiated CSCCs, and (F) poorly differentiated stroma and cancer of CSCCs.

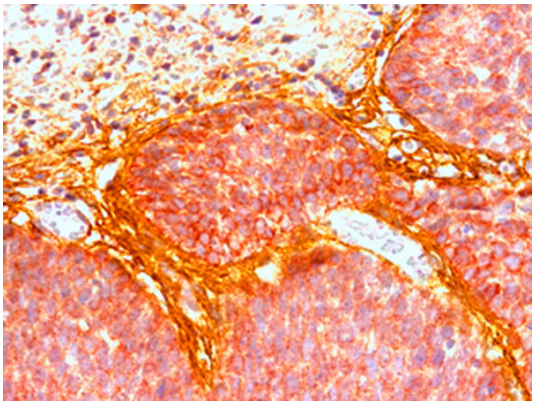

Figure 3. Images of immunohistochemical double staining for TNC (brown reaction product) in the stroma and for HPV 16/18 E6 (red reaction product) in the cancer cells of cervical cancer, respectively.

with other clinicopathological features (**Figure 3**; **Table 2**). Similarly, SMA expression was positively correlated with clinical stage (χ^2=4.498, *P*=0.034)andLVI(χ^2=11.294,*P*=0.001),butnegatively correlated with age and differentiation status. No significant association of FSP1 expression was identified (χ^2=8.359, *P*=0.004) with other clinicopathological features, except for LVI (Table S1). Data from the Cancer Genome Atlas (https://tcga-data.nci.nih.gov/tcga/) revealed that TNC, FSP1, SMA, vimentin, and CD163 were all negatively correlated with overall survival at the mRNA level (**Figure 4**). To further elucidate the association of tumor growth with TNC, SMA immunohistological double staining for TNC/CD34 and SMA/CD34 was performed. SMA expression was found in the stroma, whereas CD34 expression was observed around cancer cells. Furthermore, MVD was significantly higher in TNC (*P*=0.049) and SMA (*P*=0.032) expression-positive cervical carcinoma tissue samples than in expression-negative cases (**Figure 5**, Figure S1).

TNC expression is correlated with CAF markers in cervical cancer

To further analyze whether TNC might represent a potential CAF marker, we investigated the association between TNC and CAF markers in cervical cancer. The results showed that TNC was positively correlated with FSP1 (*r*=0.306, *P*=0.005) and SMA (*r*=0.464, *P*<0.001) in addition to vimentin (*r*=0.138, *P*=0.225). Moreover, the relationship between TNC and CAF markers was examined in individual cells of five cervical carcinoma tissues using confocal microscopy. The stromal fibroblasts were also positive for both TNC and FSP1, TNC and SMA (**Figure 6**). We noted that TAMs, as indicated by CD163 expression, were significantly more

Table 2. Relationship between TNC expression and clinicopathologic features in cervical carcinoma

Variable	*N*	TNC (-) *n* (%)	TNC (+) *n* (%)	χ^2	*P*
Age (years)				1.585	0.208
<45	44	13 (29.5)	31 (70.5)		
≥45	31	13 (41.9)	18 (58.1)		
Stage				4.945	0.026
1	42	20 (47.6)	22 (52.4)		
2-4	33	6 (18.2)	27 (81.8)		
Differentiation				1.227	0.542
Well	28	12 (42.9)	16 (57.1)		
Moderate	42	12 (28.6)	30 (71.4)		
Poorly	5	2 (40.0)	3 (60.0)		
LVI				5.920	0.015
Negative	57	24 (42.1)	33 (57.9)		
Positive	18	2 (11.1)	16 (88.9)		
HPV 16/18 E6				8.772	0.003
Negative	23	10 (43.5)	11 (47.8)		
Positive	52	14 (26.9)	38 (73.1)		

frequent in cervical carcinoma. Furthermore, CD163 was also positively correlated with TNC (r=0.279, P<0.001) (**Table 3**).

Discussion

Whereas it has been reported that TNC plays an important role in tumor progression in various cancers such as colorectal cancer [9], breast cancer [10], glioblastoma [11], glioma [12], and ESCC [6], the expression and importance of TNC in cervical carcinoma have not been well elucidated. Our results showed that TNC expression was not only more prevalent in cervical carcinoma tissues than in normal cervical tissue but was also positively correlated with SMA, FSP1, and CD163. Our findings might provide a potential diagnostic marker and therapeutic target for cervical carcinoma.

Taken together, the findings of the present study showed that TNC expression was significantly higher in cervical cancer tissues than in non-neoplastic cervical epithelium, leading to the proposal that TNC might serve as a diagnostic marker for cervical cancer. A previous study suggested that TNC expression is related to clinical stage and lymph node metastasis of ESCC stromal fibroblasts [6]. The overexpression of TNC has also been correlated with lymph node metastasis, vascular invasion, and the hepatic metastasis of pancreatic cancer [13]. This is in concordance with our result that TNC expression was positively correlated with clinical stage and LVI in the stroma of cervical carcinoma. Thus, we have also taken into account the possibility that TNC participated in the initiation, metastasis, and invasion of cervical carcinoma in our cohort.

Our study not only found that TNC was upregulated around the stroma where HPV-16/18 E6 are highly expressed in the corresponding tumor cells, but also explained a significant correlation between the expression of TNC and HPV-16/18 E6 infection. Data from the present study suggested that the increased expression of TNC is associated with HPV-induced cervical cancer and thereby might promote the initiation and progression of cervical cancer.

We also assessed the association between TNC and other tumor microenvironment-related factors such as MVD and TAM infiltration. Our results finding that TNC was positively correlated with angiogenesis in both pathological morphologies and statistical analysis for cervical carcinoma, which are in accordance with the RIP1-Tag2 tumorigenesis model, which states that TNC promotes multiple events in tumor progression and metastasis and, in particular, in angiogenesis [14]. The TNC-positive areas in macrometastases of breast cancer to the lungs have been shown to be infiltrated with myofibroblasts and macrophages [15]. Our findings are in concordance with these results in that overexpression of TNC was associated with an increased TAM population in cervical carcinoma, suggestive of myofibroblast and TAM infiltration along with TNC upregulation in areas around the cancer cells participating in the metastasis, invasion, and progression of cervical carcinoma. The current study shows that TNC expression is not only upregulated in cervical cancer but is also associated with clinical stage, LVI, MVD and TAM, consistent with the results of a retrospective study [14, 15]. Thus, our findings suggest that TNC might represent a significant biomarker for diagnosis and might be involved in the initiation of tumorigenesis and the progression to metastasis.

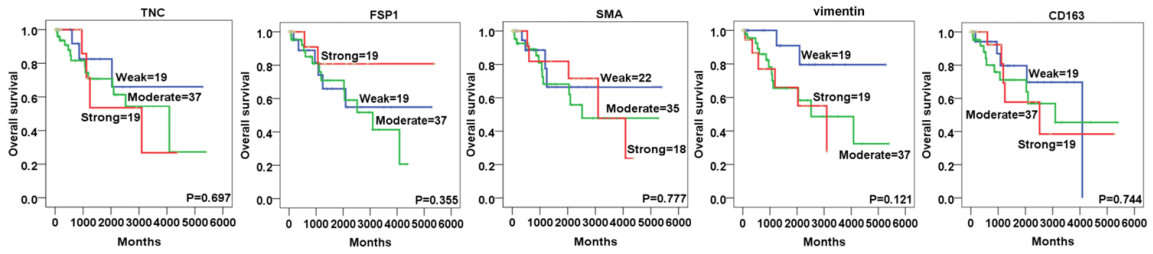

Figure 4. Kaplan-Meier analyses of overall survival curves for TNC, FSP1, SMA, vimentin, and CD163 expression in patients with cervical cancer.

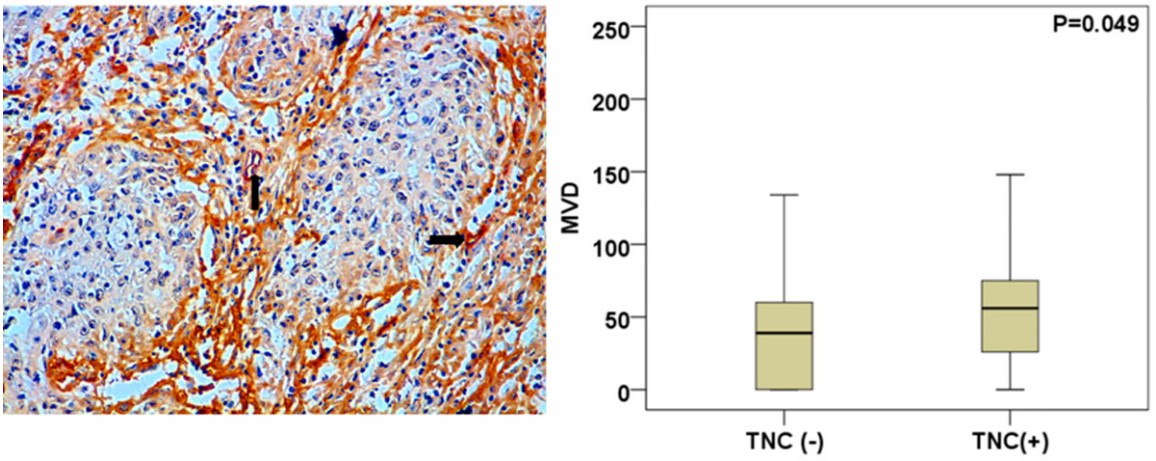

Figure 5. Images of immunohistochemical double staining in cervical cancer. Immunostaining of TNC (brown reaction product) in the stroma and CD34 (red reaction product, as indicated by arrows) around the cancer cells, respectively (left). TNC in cervical carcinoma was significantly associated with increased microvessel density (right).

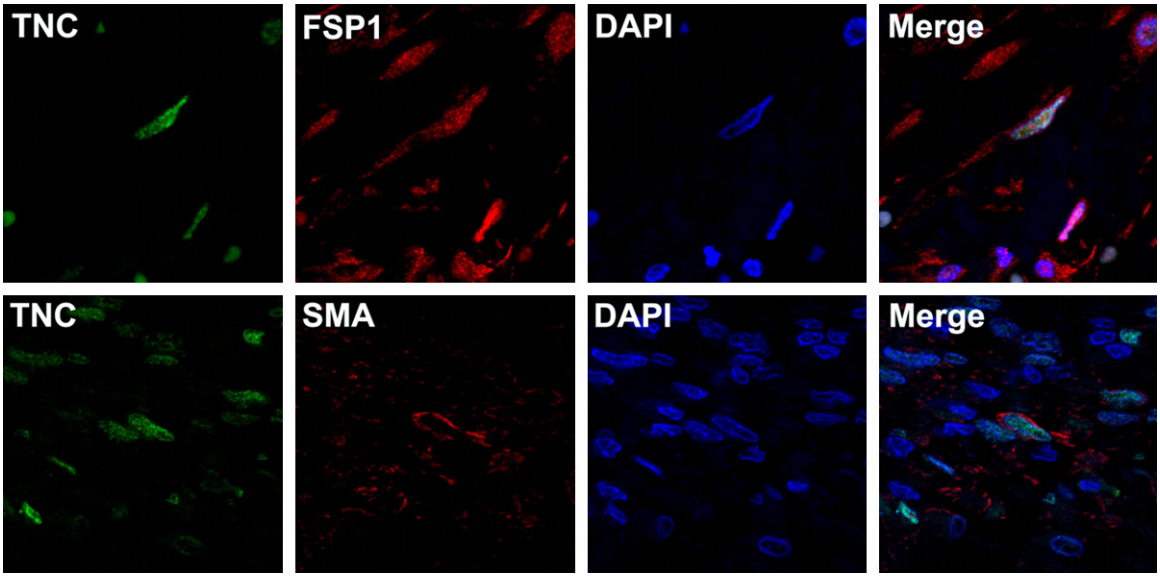

Figure 6. Immunofluorescence staining for TNC, FSP1, SMA in cervical carcinoma tissues. TNC and FSP1 protein were located in the nuclear and SMA was located in the cytoplasm of CAFs in cervical carcinoma (Green for TNC, Red for FSP1/SMA, and Blue for DAPI).

Table 3. Correlation between expression of TNC with CAF markers and CD163 in cervicalcarcinoma

Variable	*N*	TNC (-) *n* (%)	TNC (+) *n* (%)	χ^2	*P*	*R*	*Pc*
FSP1				7.859	0.005	0.306	0.003
Negative	25	14 (56.0)	11 (44.0)				
Positive	50	12 (24.0)	38 (76.0)				
SMA				20.903	<0.001	0.464	0.001
Negative	24	17 (70.8)	7 (29.2)				
Positive	51	9 (17.6)	42 (82.4)				
vimentin				1.473	0.225	0.138	0.120
Negative	28	12 (42.9)	16 (57.1)				
Positive	47	14 (29.8)	33 (70.2)				
CD163				13.040	<0.001	0.279	0.001
Negative	32	18 (56.3)	14 (43.2)				
Positive	43	8 (18.6)	35 (81.4)				

Pc: *P* value correction.

In addition, histologically immature CAFs, defined as having large, plump, spindle-shaped morphology, were associated with increased MVD, marked TAM, and a complete epithelial-mesenchymal transition phenotype [7]. CAFs support various aspects of tumor initiation, growth, and progression through the secretion of growth stimulatory, pro-survival, and angiogenic factors [16, 17]; in addition, TNC showed effects similar to those of CAFs with respect to cervical carcinoma in our study. Notably, TNC expression was not only positively correlated with the expression of CAF markers for cervical carcinoma, but TNC also co-localized with CAF markers in the same stromal fibroblasts. In particular, co-localization was observed with SMA and FSP1. It could be considered that lymph node metastasis might occur more frequently and potentially lead to poorer prognosis if TNC was positively associated with FSP1 or SMA in ESCC stromal fibroblasts; hence, TNC might serve as a useful CAF marker for the prediction of short-term survival of patients with ESCC [6]. Therefore, our study suggests that TNC represents a novel marker for CAFs in cervical carcinoma. In support of this model, immunohistochemical staining also showed high concordance and overlapping of TNC-expressing and CAF cell populations in cervical carcinoma compared to the entire stroma.

Conclusions

Taken together, our study suggests that TNC not only qualifies as a promising novel biomarker for diagnosis but also as a potential marker for CAFs in cervical carcinoma. Therefore, TNC may represent an important therapeutic target for cervical carcinoma.

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Disclosure of conflict of interest

None.

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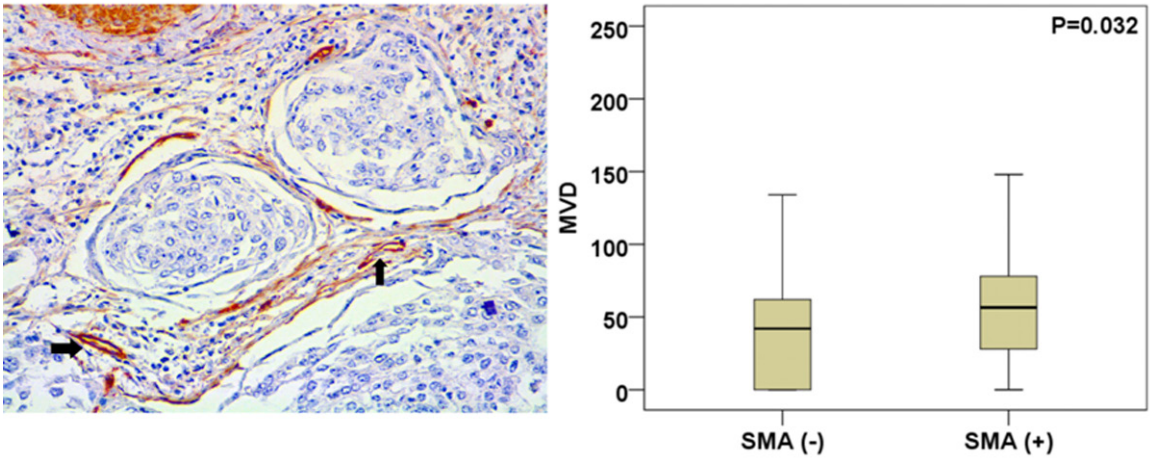
Supplementary Table 1. Association of CAF marker expression with clinicopathological characteristics

Variable	*N*	FSP1 (+) *n* (%)	χ^2	*P*	SMA (+) *n* (%)	χ^2	*P*	vimentin (+) *n* (%)	χ^2	*P*
Age (years)			0.483	0.487		0.035	0.853		1.216	0.270
<45	44	31 (70.5)			30 (68.2)			30 (68.2)		
≥45	31	19 (61.3)			21 (67.7)			17 (54.8)		
Stage			3.232	0.072		4.498	0.034		0.281	0.596
1	42	24 (57.1)			24 (57.1)			25 (59.5)		
2-4	33	26 (78.8)			27 (81.8)			22 (66.7)		
Differentiation			0.821	0.663		1.033	0.597		2.674	0.263
Well	28	17 (60.7)			17 (60.7)			21 (75.0)		
Moderate	42	29 (69.0)			30 (71.4)			24 (57.1)		
Poorly	5	4 (80.0)			4 (80.0)			2 (40.0)		
LVI			8.359	0.004		11.294	0.001		2.768	0.096
Negative	57	33 (57.9)			33 (57.9)			33 (57.9)		
Positive	18	17 (94.4)			18 (100.0)			14 (77.8)		
HPV 16/18 E6			1.087	0.297		2.055	0.152		2.901	0.089
Negative	23	14 (60.9)			13 (56.5)			12 (52.2)		
Positive	52	36 (69.2)			38 (73.1)			35 (67.3)		



Supplementary Figure 1. Images of immunohistochemical double staining in cervical cancer. Immunostaining of SMA (brown reaction product) in the stroma and CD34 (red reaction product, as indicated by arrows) around the cancer cells, respectively (left). SMA in cervical carcinoma was significantly associated with increased microvessel density (right).